

# Immune responses to soluble antigens of treponemes<sup>1</sup>

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**SUMMARY** Sonicates from five cultivable treponemes were used as antigens in delayed hypersensitivity tests and macrophage inhibition assays. Immunodiffusion analysis showed that the sonicate comprised two major antigenic components which were not distinguishable in the skin tests. The sonicate antigens elicited significant cell-mediated immunity in guinea-pigs. *Treponema refringens* biotype Nichols proved to induce the strongest delayed response. Delayed skin hypersensitivity to the antigens of *Treponema pallidum* was found in eight rabbits without orchitis, but not in six rabbits with *T. pallidum* orchitis. In contrast, the rabbits with syphilitic orchitis gave the strongest reactions with the non-pathogenic spirochetes. In terms of the cell-mediated immunity responses, *Treponema phagedenis* Reiter was found to be related to *T. phagedenis* Kazan 4 and *Treponema denticola*. *Treponema scoliodontum* was related to *T. phagedenis* Kazan 5, and *T. refringens* biotypes Nichols and refringens. The antigens of *T. pallidum* had the closest relationship to *T. refringens* biotypes refringens and Nichols, *T. phagedenis* biotype Reiter, and *T. scoliodontum*. It was also demonstrated that some three of 12 human syphilitic sera reacted with the antigens of *T. pallidum* but not with control sera.

## Introduction

The dermal hypersensitivity to treponemal antigens in man is associated with latency but not with the primary stage of syphilis (Musher and Schell, 1974); the migration of leucocytes obtained from late latent stage is inhibited by proteins of Reiter treponemes, whereas the migration of leucocytes from primary syphilis is enhanced (Fulford and Brostoff, 1972); the blastic transformation of lymphocytes by treponemal antigens and Reiter protein is greatest in secondary and least in early primary syphilis (Bădănoiu *et al.*, 1969; Fulford and Brostoff, 1972).

However, little is known about the effect of treponemal antigens on cell-mediated immunity and the role they play in the biological processes involving them. We attempted to find out if soluble antigens of treponemes can elicit cell-mediated immunity and whether these antigens obtained from different treponemes are related to each other and to *Treponema pallidum*.

## Materials and methods

### MICRO-ORGANISMS

The following strains of non-pathogenic treponemes were used: *Treponema phagedenis* biotype English Reiter (obtained from Dr A. W. Hanson, the Center for Disease Control, Atlanta, Georgia). *T. phagedenis* biotype Kazan 4, *T. phagedenis* biotype Kazan 5, *Treponema refringens* biotype refringens, *T. refringens* biotype Nichols, *Treponema denticola*, and *Treponema scoliodontum* (obtained from Dr R. C. Johnson, The University of Minnesota, Minneapolis, Minnesota). The pathogenic *T. pallidum* was received live in rabbits' testicles (obtained from Dr J. N. Miller, The University of California, Los Angeles).

The non-pathogenic treponemes were grown in Hanson and Cannefax's (Hanson and Cannefax, 1964) modified liquid medium consisting of 45% thioglycollate broth by volume (BBL), 45% brain heart infusion by volume (BBL), 0.05% sodium thioglycollate mass/vol. (Difco), and 10% normal rabbit serum by volume inactivated at a temperature of 56°C for 30 minutes. 400 ml of the medium was dispensed into screwcapped bottles, and inoculated with 10 ml of a heavy growth culture. The inoculated media were incubated at a temperature of 37°C for a week until heavy turbidity appeared.

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## PREPARATION OF ANTIGENS FROM SAPROPHYTIC TREPONEMES

The saprophytic treponemes were grown until heavy turbidity was observed. Pure cultures of treponemes were treated with 0.2 ml of 1% sodium azide, and left at room temperature overnight. The killed treponemes were centrifuged at 23 500 *g* for 15 minutes in an International refrigerated centrifuge model B-20 (International Equipment Co., Mass., USA). The sediment was washed six times with a 0.85%, pH 7.2 phosphate buffered saline (PBS) until no trace of protein was left in the washings as measured at 280 nm wavelength in a Unicam 1800 ultraviolet spectrophotometer. To the final washings, five times volume of a  $10^{-5}$  mol/l, pH 7.4 EDTA (ethylene diaminetetraacetic acid) solution containing 100 units penicillin and 50 µg streptomycin per ml was added, and the suspension was sonicated in a Biosonik III ultrasonicator for 20 minutes at 20 kHz/s, or until at least 80% of the treponemes were disrupted. The cell debris and cytoplasmic membranes were then removed by centrifugation at 23 500 *g* for 20 minutes. The supernatant was collected and recentrifuged at the same speed for 10 minutes one or more times, until no particulate material was found microscopically at  $\times 1000$  magnification in smears prepared from the supernatant and stained by Fontana-Tribondeau's method. This fluid representing the cytoplasm was lyophilised.

PREPARATION OF ANTIGENS FROM *T. PALLIDUM*

All rabbits were first examined physically for possible presence of skin lesions, and their sera were examined by the Venereal Disease Reference Laboratory (VDRL) test, to ensure that they did not contain reagins.

Male albino rabbits weighing about 3.6 kg (8 lb) and found free of skin lesions and reagins were injected with 0.5 ml of a suspension containing  $10^8$  live cells of *T. pallidum* into each testicle. The animals were isolated in rooms at a temperature of 18–20°C. When orchitis developed on the 18th to 22nd day, the rabbits were bled, and the testicles removed. The testicular glands were then excised from the overlying tissues, placed in a triple volume of a 0.05 mol/l, pH 7.4 PBS containing 1.3 mol/l sucrose, homogenised in a Sorvall omni-mixer at the lowest speed for 30 seconds, and centrifuged at 900 *g* to remove cell debris. The supernatant was collected and centrifuged for one hour at 17 000 *g*, to separate treponemes from fine cell debris. The supernatant, containing treponemes was then collected and dialysed overnight against 0.05 mol/l, pH 7.4 PBS. The dialysate was concentrated to 1/10 original volume of the tissue suspension. The partly purified tre-

ponemes kept on crushed ice were then disrupted by ultrasonication at 20 kHz/s for three minutes. The material thus obtained was centrifuged at 760 *g* for 10 minutes, and the supernatant was gently withdrawn and recentrifuged as above. The final supernatant representing partly purified antigens of *T. pallidum* was filtered through a 0.45 µm membrane filter. Each step of this preparation technique was checked by withdrawing aliquot samples and examining them at  $\times 600$  magnification by dark field microscopical examination and at  $\times 1000$  magnification in smears stained by Fontana-Tribondeau's method.

In order to obtain a control material, normal, non-injected healthy rabbits were bled; the testicles were removed and the fatty tissue was removed. The defatted testicles were homogenised in a 0.05 mol/l, pH 7.4 PBS, by means of a Sorvall omni-mixer and processed further as described above.

## THE SKIN HYPERSENSITIVITY ASSAY

Albino, randomly bred guinea-pigs, weighing from 600 to 800 g were used throughout the studies, after their sera had been examined by the VDRL test to ensure that they did not contain reagins.

The guinea-pigs were injected into the hindfoot pads with 0.1 ml of a solution containing 300 µg/ml of a lyophilised extract of the saprophytic treponemes made in a  $10^{-5}$  mol/l, pH 7.4 EDTA supplemented with 100 unit penicillin and 50 µg streptomycin per ml. Twelve guinea-pigs were used for each strain of treponeme, making a total of 84 guinea-pigs employed for studies obtained from seven different types of saprophytic treponemes.

For immunological assays on *T. pallidum*, New Zealand albino rabbits received injections of 0.5 ml of PBS suspensions containing  $10^8$  live cells of *T. pallidum* into each testicle. The infected rabbits that showed signs of orchitis and those that appeared to be free were then subjected to dermal challenges as described below.

Before the assay, all the antigen preparations were examined for possible toxic or allergic effect, by injections of 500–600 µg of each material into the skin of normal guinea-pigs; but none showed any skin or general reaction.

For the intradermal challenge, sterile solutions containing 100 µg/ml of the lyophilised sonicates reconstituted with  $10^{-5}$  mol/l, pH 7.4 EDTA, supplemented with penicillin and streptomycin were used.

Groups of four guinea-pigs sensitised with an individual antigen preparation were taken for the dermal hypersensitivity assay 12, 20, and 30 days after first injection. The skin on the back was first shaved and short hair was then removed with 'Neet' (Whitehall Laboratories Limited, Toronto). 0.1 ml

volumes of different antigens were then injected into different sites. 0.1 ml of saline and culture medium were used as controls. The test sites were examined at 24, 48, and 72 hours observing the appearance of erythema and measuring the dimensions of indurations in two perpendicular directions. Since indurations had irregular shapes, the measurements were standardised by multiplying the longest by the shortest axis, yielding a square of the induration area.

#### MACROPHAGE MIGRATION INHIBITION (MMI) ASSAY

The actual MMI assays were preceded by a procedure used to ensure that the sonicated antigens were not harmful to macrophages. For this purpose, peritoneal macrophages obtained from normal guinea-pigs, were exposed to sterile solutions of the sonicates (300 µg/ml) for one hour at a temperature of 37°C. The viability of the macrophages was determined microscopically upon staining with 0.1% trypan blue.

In order to obtain sensitised macrophages for the assays, groups of three guinea-pigs were first injected with 10 µg of the lyophilised sonicates, reconstituted in a 10<sup>-5</sup> mol/l, pH 7.5 EDTA containing penicillin and streptomycin, or with sterile PBS. On the 20th day the animals were examined for dermal hypersensitivity, and only those reacting with the corresponding antigens (except guinea-pigs injected with PBS) were used as donors of macrophages. The guinea-pigs to be used as donors received intraperitoneally 30 ml of a sterile 2.5% soluble starch gel (Difco, Michigan). After three days the animals were bled out by cardiac puncture. The peritoneal cavity of guinea-pigs was rinsed with 100 to 250 ml of sterile Hanks's solution, and the liquid was aspirated and centrifuged in silicone-coated glass tubes for 10 minutes at a temperature of 4°C in an IEC international centrifuge (Universal Model UV) at 1200 rev/min. The sedimented cells were washed twice with sterile Hanks's BSS and recentrifuged for five minutes at 1000 rev/min at a temperature of 4°C. The washed peritoneal cells were suspended by adding 0.1 ml of packed cells to 0.5 ml of the tissue culture medium 199 containing 15% normal guinea-pig serum. The cell suspension was kept in an icebath to minimise the cellular metabolism. Before use, 50 µl capillary tubes were filled with the cell suspension, sealed at one end with a non-drying modelling clay (Peter Austin MFG Co., Toronto), and spun for five minutes at 900 rev/min, at room temperature in an IEC centrifuge. The capillary tubes were then cut below the packed cell-liquid interface and fastened individually inside a plastic incubating chamber (1.5 cm diameter, 0.2 cm

depth) with silicone grease. The chambers were filled with 0.6 ml of the tissue culture medium 199 containing 15% normal guinea-pig serum and 0.1 ml of a treponemal sonicate (100 µg/ml of 10<sup>-5</sup> EDTA containing penicillin and streptomycin). The chambers were then covered with a sterile cover glass, sealed with grease, and incubated at a temperature of 37°C for 24 hours. The area of migration was mapped by projection microscopy (Nikon Profile Projector, Model 6 C, Nippon Kogaku K.K., Japan) and measured by planimetry (K + E, model 62002, Keuffel and Esser Co., Germany). The macrophage migration inhibition (MMI) indices were expressed as shown below:

$$\text{MMI} = 1 - \left( \frac{\text{area of migration with antigen}}{\text{area of migration without antigen}} \right) \times 100$$

The final MMI index for an individual cytoplasm was calculated by averaging the measurements taken from at least four chambers.

#### IMMUNODIFFUSION PROCEDURES

The treponemal antigens were also examined against antisera produced in rabbits by serial injections of the sonicates (Kwapinski, 1972) and against six sera obtained from rabbits injected with live *T. pallidum* as described above. The following assays were employed: an immunodiffusion test set up in 1% agarose made in a 0.06 mol/l, pH 8.2 PBS, and a counterimmunoelectrophoresis run in 1% Indubiose (Remington *et al.*, 1972). When a cross-reaction was noted the antiserum was absorbed with a heterologous antigen preparation in the following manner: 1 ml of a sonicate containing 200 to 300 µg of dry weight was added to 1 ml of an antiserum, or globulin solution, incubated for 30 minutes at a temperature of 37°C in a shaker bath, and centrifuged for 20 minutes at 19 000 g. The supernatant was collected, recentrifuged as above, and brought to the original volume of the globulin solution by vacuum dialysis at 3°C. The absorbed globulins were then re-examined against both the homologous and heterologous antigen preparations and were used for further tests if no reaction with the heterologous antigens was observed.

#### STATISTICAL EVALUATION METHODS

The final data analysed consisted of various measurements made for each combination of challenge and source antigen at three postsensitisation times (12, 20, 30 days). The measurements recorded were the square of the induration area 24 and 48 hours after the test site was cleared for the hypersensitivity assay, and the macrophage migration inhibition 24 hours after incubation for this assay.

These data were analysed by undertaking a two-way analysis of variance on each measurement, the seven antigen sources being one classification, the seven challenge antigens, the other. The order of presentation of antigen sources was randomised for an individual challenge antigen.

If significant differences were found between challenge antigens, Duncan's multiple range test (Bruning and Kintz, 1968) was used by making individual comparisons to determine immunological relationships.

## Results

### THE DELAYED HYPERSENSITIVITY ASSAY

The delayed dermal hypersensitivity lesions appeared as erythema and oedema, with occasional necrosis. Histologically, the affected sites showed infiltrations consisting predominantly of mononuclear cells, including macrophages with polymorphonuclear leucocytes found mainly at earlier stages of the response.

The data obtained from all the delayed hypersensitivity reactions, pooled, and expressed as the average of induration elicited by the antigens of each treponemal strain are shown in Table 1. It is apparent that the antigens of *T. refringens* biotype Nichols induced the most intense hypersensitivity, whereas those of *T. scoliodontum* proved to be the weakest in this respect.

In rabbits showing orchitis after intratesticular injections with live *T. pallidum*, the challenge with partly purified antigens of *T. pallidum* did not evoke any skin reaction. In contrast, delayed dermal hypersensitivity responses were observed in eight rabbits that failed to show signs of overt orchitis but had an apparent latent infection as shown by strong

VDRL reactions. These rabbits challenged with the antigens of *T. pallidum* on the 60th to 80th day after intratesticular injections of live *T. pallidum* showed areas of induration and necrosis at the injection site measuring 16 to 18 mm<sup>2</sup> in 48 to 72 hours. No reaction to the challenge with extracts obtained from normal testicles was ever noted.

Antigens of the saprophytic treponemes introduced into the skin of six rabbits showing syphilitic orchitis, but not in rabbits with a latent infection, elicited skin hypersensitivity reactions with the average induration areas (mm<sup>2</sup>) ranging from 31 to 146 in 24 to 48 hours, as follows: *T. phagedenis* biotype English Reiter (65.0), *T. scoliodontum* (53.0), *T. phagedenis* biotype Kazan 5 (49.0), *T. denticola* (31.0), *T. phagedenis* biotype Kazan 4 (14.6), *T. refringens* biotype Nichols (10.0), and *T. refringens* biotype refringens (10.0). No skin reaction was caused when these antigens were injected into the skin of normal (non-sensitised) rabbits.

Results obtained from a total of 190 hypersensitivity tests carried out in guinea-pigs sensitised with the antigens of an individual treponemal strain and challenged with the antigens of different treponemes are shown in Table 2. The statistical analysis was carried out by a programme of factorial design with an error of 0.31 and the standard error of square equal to 0.01. Saline and culture medium controls gave readings of erythema, but no induration of 0.76 to 0.80 mm<sup>2</sup>.

For the assessment of relationships between the soluble antigens of different treponemes by Duncan's test only the data obtained on the 20th and 30th day after sensitisation were used because those read on the 12th day were less distinct. Each antigen was assumed to be 100%, and simple percentage calculations were employed for the determination of

Table 1 Dermal hypersensitivity readings in guinea-pigs sensitised with a single treponemal sonicate and challenged with antigens of different spirochetes

Challenge antigens of:	Mean area* (mm <sup>2</sup> ) of skin induration in guinea-pigs sensitised with antigens of						
	<i>T. scoliodontum</i>	<i>T. phagedenis</i> English Reiter	<i>T. phagedenis</i> Kazan 5	<i>T. phagedenis</i> Kazan 4	<i>T. denticola</i>	<i>T. refringens</i> Nichols	<i>T. refringens</i> refringens
<i>T. scoliodontum</i>	38.9	28.0	14.8	19.7	19.5	22.8	27.5
<i>T. phagedenis</i> , English Reiter	29.4	58.7	30.5	28.1	37.0	31.8	26.5
<i>T. phagedenis</i> Kazan 5	25.1	36.3	37.7	29.8	37.3	36.8	26.3
<i>T. phagedenis</i> Kazan 4	30.2	35.3	32.7	42.4	40.3	41.3	25.5
<i>T. denticola</i>	29.1	34.0	37.5	30.1	57.9	48.0	26.2
<i>T. refringens</i> Nichols	24.9	32.2	32.2	31.7	37.8	87.6	38.4
<i>T. refringens</i> refringens	23.2	31.6	29.0	36.5	33.7	69.9	49.9

\*Results read after 24 and 48 hours, pooled and evaluated.

Table 2 Results of the delayed hypersensitivity assays read on challenge on the 20th day (left) and 30th day (right) after sensitisation with treponemal antigens and expressed by comparative percentages

Antigen	Day 20							Day 30						
	1	2	3	4	5	6	7	1	2	3	4	5	6	7
1. <i>T. scoliodontum</i>	100	44	90	51	48	37	92	100	88	37	58	84	66	58
2. <i>T. phagedenis</i> biotype English Reiter	25	100	3	75	91	83	10	88	100	10	74	97	59	74
3. <i>T. phagedenis</i> biotype Kazan 5	90	49	100	61	53	41	97	42	47	100	60	49	28	60
4. <i>T. phagedenis</i> biotype Kazan 4	21	79	39	100	87	66	34	70	79	34	100	81	46	100
5. <i>T. denticola</i>	5	91	15	86	100	76	9	85	96	3	78	100	57	78
6. <i>T. refringens</i> biotype Nichols	31	81	43	50	70	100	50	50	31	55	13	55	100	13
7. <i>T. refringens</i> biotype refringens	93	47	97	57	52	76	100	70	79	34	100	81	46	100

comparative percentages; a level at, and above, 70% was arbitrarily selected as a cut-off for meaningful antigenic relationships.

#### MACROPHAGE MIGRATION INHIBITION TEST

Within the limits of the statistically significant results computed from 96 readings made for each source of antigen, the antigens of *T. denticola* caused the greatest and *T. refringens* biotype refringens the smallest migration inhibition of all sensitised macrophages (Table 3). Migration areas of the macrophages obtained from normal peritoneal exudates and exposed to the antigens of saprophytic treponemes were found to range from 4.7 to 5.2 cm<sup>2</sup>.

Indices of macrophage migration inhibition were found in most cases to increase with the length of time passed after the initial sensitisation of guinea-pigs with the antigens. For example, MMI indices in respect to *T. phagedenis* biotype Kazan 4 varied from 5% after 12 days, 22% after 20 days, to 39% after 30 days; and MMI indices in respect to *T. scoliodontum* increased from 19% after 12 days to 36% after 20 days, and 33% after 30 days. The data obtained from MMI tests carried out with the

macrophages derived from guinea-pigs after 20 to 30 days past the sensitisation were employed for the determination of mutual immunological relationships between the treponemes.

The analysis of Duncan's new multiple-range test and calculations of comparative percentages from the averaged data obtained when macrophages were received at the 20th day after sensitisation (Table 4) revealed significant similarities existing between the following pairs of treponemes: *T. phagedenis* biotype Kazan 5 and *T. phagedenis* biotype English Reiter (comparative percentages 86 and 85), *T. denticola* and *T. phagedenis* biotype English Reiter (76 and 70), *T. denticola* and *T. phagedenis* biotype Kazan 5 (88 and 88), and *T. refringens* biotype Nichols and *T. scoliodontum* (comparative percentages 98 and 99) and perhaps *T. denticola* and *T. scoliodontum* (65 and 73). No relationships were discovered, by this test, between other treponemes, since their corresponding comparative percentages fell below the 70% cut-off line.

When macrophages obtained on the 30th day after sensitisation were employed, statistically significant differences ( $\alpha = 0.05$ ) were observed between the following pairs of treponemes: *T.*

Table 3 Relative macrophage inhibitions caused by treponemal antigens

Macrophage sensitised with antigens of	Relative macrophage inhibition caused by antigens of:						
	<i>T. scoliodontum</i>	<i>T. phagedenis</i> biotype English Reiter	<i>T. phagedenis</i> biotype Kazan 5	<i>T. phagedenis</i> biotype Kazan 4	<i>T. denticola</i>	<i>T. refringens</i> biotype Nichols	<i>T. refringens</i> biotype refringens
<i>T. scoliodontum</i>	33	22	24	25	0	0	61
<i>T. phagedenis</i> biotype English Reiter	22	40	31	35	5	3	26
<i>T. phagedenis</i> biotype Kazan 5	10	27	37	30	0	4	18
<i>T. phagedenis</i> biotype Kazan 4	30	37	39	37	0	5	64
<i>T. denticola</i>	13	22	34	31	19	0	47
<i>T. refringens</i> biotype Nichols	23	27	38	11	0	14	51
<i>T. refringens</i> biotype refringens	40	25	38	18	0	0	60

Table 4 Comparative percentages calculated on results obtained from macrophage migration inhibition assays carried out with macrophages withdrawn at 20 days after sensitisation with treponemal antigens

Antigen	1	2	3	4	5	6	7
1. <i>T. scoliodontum</i>	100	56	65	7	73	99	39
2. <i>T. phagedenis</i> biotype English Reiter	23	100	85	44	70	20	70
3. <i>T. phagedenis</i> biotype Kazan 5	47	86	100	2	88	44	60
4. <i>T. phagedenis</i> biotype Kazan 4	51	29	33	100	37	52	20
5. <i>T. denticola</i>	65	76	88	36	100	62	54
6. <i>T. refringens</i> biotype Nichols	98	55	64	8	72	100	39
7. <i>T. refringens</i> biotype refringens	51	59	36	12	15	49	100

*denticola* and *T. scoliodontum* (comparative percentages 84 and 86), and *T. denticola* and *T. phagedenis* biotype Kazan 5 (79 and 75). Comparative percentages between all other treponemes were found to fall below the 70% limit.

A confrontation of the data obtained by the two assays used for measurements of cell-mediated immunity showed a 54.76% correlation in homologous systems and 21.43% in heterologous systems, if both tests were positive. They also coincided in 8.33% and 30.55% of negative findings, respectively. In homologous systems, 29.77% results were positive by dermal hypersensitivity reaction and negative by MMI test, and 7.14% were positive by MMI while being negative by dermal hypersensitivity assay. In heterologous systems, 30.55% results were positive by skin test while being negative by MMI test, and 17.27% were positive by MMI while being negative by the dermal hypersensitivity assay.

#### IMMUNODIFFUSION TESTS

The antigens of treponemes produced two precipitation lines on reaction with homologous antigens but only a single band when a reaction occurred in a heterologous system. Cross-reactions were found mainly between the antigens of *T. pallidum*, *T. refringens* biotype Nichols, *T. phagedenis* biotype Reiter, *T. refringens* biotype refringens, *T. denticola* and *T. scoliodontum*, as well as between *T. phagedenis* Reiter, *T. refringens*, *T. denticola* and *T. scoliodontum*, between *T. refringens* Nichols and *T. denticola*, and between *T. phagedenis* Kazan 4 and *T. refringens*.

Tentative studies performed with the treponemal antigens and human sera from 12 cases of primary syphilis revealed antibodies reacting with the antigens of *T. pallidum* (three sera) and *T. phagedenis* biotype English Reiter (three sera, two of which also reacted with *T. pallidum*). No reactions with these antigens were given by 12 normal sera and 12 sera obtained from diseases such as leukaemia, fungal dermatitis, and pneumonia.

The sera of all 12 rabbits that showed orchitis and the sera of all eight rabbits that did not show orchitis in 40 to 60 days after injections of live *T. pallidum*, were found to react with the antigens of *T. pallidum* forming one or two bands on the immunoelectrophoresis. These sera examined before the injection of treponemes did not react with *T. pallidum* antigens and were VDRL-negative. Regardless of the presence or absence of orchitis, the sera obtained after 25 to 40 days, respectively, reacted with cardiolipin at final dilutions ranging from 1:16 to 1:64, as examined by Card test.

#### Comments

The antigens present in sonicates of the treponemes appear to be potent antigens, capable of eliciting significant cell-mediated immunity in guinea-pigs. Although the exact immunochemical nature of the antigens has not been elucidated, it seems that one of the two major antigenic constituents is shared by a number of known species of treponemes, including *T. pallidum* and *T. refringens* biotype Reiter. Antigenic relationships between the various treponemes, revealed by cellular immunity assays, seem to be more complex than those shown by immunodiffusion procedures, and they coincide with the findings reported by other investigators (Turner and Hollander, 1957; Cannefax and Garson, 1959; Pillot and Faure, 1959; Meyer and Hunter, 1967; Pillot, 1969).

The cell-mediated immunity assays show that the treponemes may tentatively be divided into two groups as follows: Group A consisting of *T. phagedenis* Reiter, *T. phagedenis* Kazan 4, and *T. denticola*, and Group B composed of *T. scoliodontum*, *T. phagedenis* Kazan 5, and *T. refringens* biotypes Nichols and refringens.

The antigens of *T. pallidum* appear to be most closely related to those of *T. refringens* biotypes refringens and Nichols and *T. phagedenis* biotype Reiter, and *T. scoliodontum*. The observation that some syphilitic sera from patients and all sera of

rabbits infected artificially with *T. pallidum* react with the partly purified antigens of *T. pallidum* suggests that thoroughly purified cytoplasmic antigen(s) of *T. pallidum* may prove to be useful as a diagnostic immunological tool.

A technical detail that deserves mentioning here is the fact that we were able to grow *T. pallidum* successfully in testicles of rabbits kept at room temperature (about 18°C). At this temperature, orchitis and a great multiplication of the treponemes occurred regularly although a little more slowly than they did at 22°C (which had commonly been regarded as a precondition for the growth of *T. pallidum*).

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